THE GATEWAY TO DIFFICULT SEPARATIONS, SELECTIVITY OR EFFICIENCY? PART II

Separations Scientists are facing an increasing number of alternative HPLC Columns that are offering either orthogonal selectivity or extremely high plate numbers (efficiency) compared to those available say, 5 years ago. When faced with new separations that need to be performed, and in many cases these are getting more complex, which route do they opt to pursue at the method development stage.

Much recent commercial activity has seen the promotion of ultra high efficiency columns, either by virtue of small particle sizes or use of elevated temperature (or both) as an option but we also have some unique novel chemistry, which are designed to offer alternate selectivity to the 'traditional' end-capped C18 (L1 designation in the US Pharmacopoeia).

In the first part of the article [1] the benefits of utilising the selectivity route were discussed, here we examine the issues surrounding the 'plate count' route.

THEORY

Since the aim of the exercise here is to obtain optimum separation between peaks (and the definition varies from one application to another) it is important to recognise the effect that plate count, resolution and speed of analysis have on each other from a purely theoretical viewpoint.

If only that were the end of the matter then it would merely be a case of using the most efficient columns on the market from the column supplier who can make the smallest particles. However as we shall see, there are practical issues with Instrumentation, which limit the particle sizes, which can be accommodated with current technology since Pressure across the column varies inversely as to the square of the particle diameter;

$$P \propto F/ d_p^2 \qquad Eqn.3$$

Also increasing speed (*F* in Eqn. 3 is the flow rate in ml/min) will also increase the pressure linearly thus the system as a whole can limit the theoretical maximum efficiencies from being attained.

BACKGROUND

Since the particle size determines the ultimate plate count that may be achieved we shall consider particle size and efficiency, relatively speaking, as one in the same.

Early researchers in the field of particles for LC use such as Knox, Huber, Unger and others concluded in the early 1970's that the way to make separations faster and more efficient was to reduce the d_p of the packings.

As far back as 1975 predictions were being made by Halasz [2] regarding the ultimate limits that could be achieved in HPLC which were pretty much the cornerstone of what was accepted as the 'status quo'. Halasz said that at column pressures of 500 bars the temperature of the eluent might increase up to 35 degrees celcius.

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Chromatography Focus

This can be seen from the Purnell equation thus;

$$R = 4\sqrt{N}[(\alpha-1)/\alpha][k/(1+k)], \qquad Eqn.1$$

where *N* is the efficiency, α is the separation factor and *k* is the retention factor (can be viewed as the loose 'speed ' term for this context)

The resolution is proportional not linearly to the plates available but to the square of the efficiency term thus making the maximisation of the plates even more important to improve the separations. Since the efficiency is related to the particle diameter thus;

$$N = L/H \qquad Eqn.2$$

Where *H* is the height equivalent to a theoretical plate; a measure of the measured column efficiency; HETP = L/N, where *L* is column length and *N* is the number of theoretical plates. HETP should be approximately 2–3 d_p for 5µm particles with a typical well-packed HPLC column; HETP (or *H*) values are usually in the range of 0.01-0.03 mm. It indicates the distance an analyte moves while completing one adsorption/desorption step between the mobile and stationary phases. This leads to the relationship that the lower the particle diameter the higher the efficiency of the column as *H* becomes smaller i.e. the HETP term dictates that more plates are present in a column of fixed length, the smaller the d_p.

A further benefit is that the optimum efficiency for a given particle size is reached at a higher flow rate *(see Figure 1)* thus giving additional benefit of being able to use high speed without loosing efficiency.

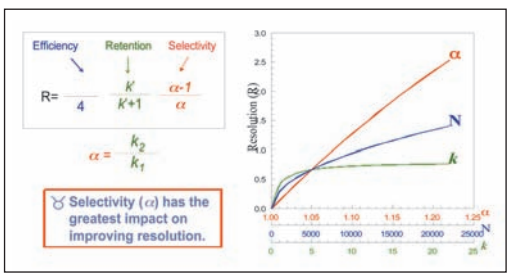

Figure 1. Factors affecting resolution.

This was due to temperature and viscosity gradients existing in both axial and radial directions inside the column. For routine work the dip should be 5µm < d_p <3µm and the minimum d_p is between 1 and 2µm. He also recommended that the maximum pressure of an LC system should be approx. 400 bars.

The technical barriers that had to be overcome (at that time 10µm irregular particles were the benchmark) to produce the micro particulate particles on a regular basis were novel synthesis protocols, novel sizing techniques and advances in column packing technologies. These in turn [3] would allow:

- higher resolution as a result of maximising column efficiency
- significant reduction of analysis time
- enhanced sensitivity
- reproducibility, robustness and ruggedness to be maintained, to become available to separation scientists.

The methods employed to achieve these goals were,

- Reduction of particle size of the packing
- Decrease column lengths and i.d.
- increase the column pressure and temperature.

Or, as was to be the case, a combination of all 3. The particle size issue was viewed as the main hurdle to overcome as it has been demonstrated to have the greatest effect on efficiency.

Table 1 shows the plates per meter for various micro porous particle sizes.

Although columns became more robust for each particle size during the life times of the size considered to be `maximum efficiency columns' it was not until the launch of the ACQUITY UPLC® (Ultra Performance Liquid Chromatography) system by Waters Coroporation in 2004.

Particle size/µm	Year of introduction	N/m Range
10	Early 1970`s	30,000 - 45,000
5	0.010 mid 1970`s	80,000 - 100,000
3	0.006 early 1980`s	100,000 - 160,000
1.7	2004	225,000 - 250,000

PRACTICAL CONSIDERATIONS

In order to realise the maximum efficiency these columns are capable of generating the Instrument (and silica particles) utilised must not only be capable of pressures above the conventional 400 bar but dispersion of the system must be kept ultra low with high quality engineering and design components mandatory. The total dispersion, or band broadening, σ^2 SYSTEM is constant for a given system and the measured column efficiency is a function of σ^2 TOTAL. As the column i.d. is decreased as with most of the columns containing sub 2µm particles, σ^2 COLUMN is reduced and the σ^2 SYSTEM dominates. Therefore σ^2 SYSTEM must be minimised to achieve maximum efficiency with reduced-bore (e.g. 2mm i.d.) columns. σ^2 SYSTEM consists of the connection tubing, interstitial column volume and the detector cell. Again the detector cell must be of minimal dispersion but also the rise time of the detector must be capable of recognising the speed at which the narrow peaks elute from the column.

Vastly improved efficiency can be obtained with a traditional 150x4.6mm 5µm column of say 90,000 plates/m when used with a conventional 400 bar system and associated plumbing and σ^2 SYSTEM . Running this column on a UPLC system can add another 25% in terms of plates to the reported efficiency.

Sub 2µm particles are a very good filtration bed and easily block up especially with bacteria that grows within buffers if these are not regularly changed. In order to realise the benefits from these ultra efficient columns ultra care needs to be paid to 'lab house keeping' that was maybe taken for granted somewhat with the slow increase, until 2004, of the efficiencies of the columns on the market.

Several Instrument manufacturers now offer commercial UPLC alternatives and columns to accompany the systems.

Almost all have a software utility that allows the scientist to calculate important parameters and migrate their analyses from HPLC to UPLC.

What needs to be remembered is that most of them assume the same physical and chemical characteristics are a function of the particles in the HPLC Column as the UPLC Column which is not always the case. Many scientists now develop new assays directly onto UPLC systems and this trend will probably increase as the acceptance of the UPLC technology into mainstream work increases.

Figure 2 shows how methods can be transferable with dramatic savings on run times.

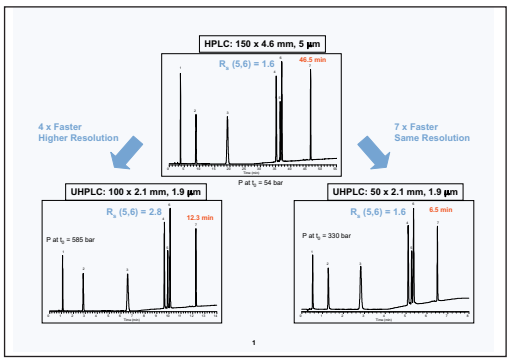

Figure 2. HPLC to UHPLC

Here Ibuprofen and some impurities are run on a column containing 5µm particles and dimensions of 150x4.6mm with good separation and a resolution between peaks 5 and 6 of 1.6 and total run time of 48 mins. For the method to be truly transferable then so long as the ratio of column length to particle size remains constant the efficiency improvement should prove beneficial with regards to speed, or resolution (operators decision). By transferring to a 50x2.1mm UHPLC column containing 1.9µm particles the analysis time is reduced by a factor of seven yet the resolution is maintained at 1.6 for peaks 5 and 6. If increased resolution is required then a longer column, in this case 100x2.1, is required *(see Figure 1)* at a trade off of total run time (doubles) then a value of 2.8 is observed. Never the less the experiments show that substantial time (and solvents – see *Figure 3*) can be saved by utilising columns with small particle size and shorter lengths.

Original method Column I : 150 x 4.6 mm, 5 µm Flow rate – 1 mL/min (Column volume = 1.7 mL) Injection volume = 10 µL				UHPLC Column II : 100 x 2.1 mm, 1.9 µm Flow rate – 0.55 mL/min (Column volume = 0.24 mL) Injection volume = 1.4 µL			UHPLC Column III : 50 x 2.1 mm, 1.9 µm Flow rate – 0.55 mL/min (Column volume = 0.12 mL) Injection volume = 0.7 µL		
# of column volumes	%B	Volume of mobile phase (mL)	Gradient time (min)	Volume of mobile phase (mL)	Gradient time (min)	%B	Volume of mobile phase (mL)	Gradient time (min)	%B
0	0	0	0	0	0	0	0	0	0
14.7	0	25	25	3.5	6.4	0	1.8	3.2	0
32.4	85	55	55	7.8	14.1	85	3.9	7.1	85
41.2	85	70	70	9.9	17.9	85	4.9	8.9	85

Figure 3. Conditions used for method transfer.

CONCLUSIONS

Although we have painted a picture where it appears that the choice is one or the other criteria, the reality is that the resolution equation contains components related to selectivity and efficiency (plates) so it would be foolhardy to ignore one completely in favour of the other. The future direction is best served with highly efficient columns in a wide range of column chemistries. There is still the issue regarding the need for specialist instrumentation and more diligent sample preparation when adopting the position of maximum plate count due to the pressures involved which may preferentially move scientists in a particular direction.

If we look 5 years down the line it is pretty certain that highly efficient columns of > 200,000 plates/m will become standard, the uptake will be greater and faster if column selectivity is taken into consideration and Instrumentation costs and reliability are acceptable in the customers eyes and pockets.

For more detailed opinion and thoughts, readers are directed to an article by Prof Klaus Unger and co-workers [4].

REFERENCES

[1]. International Labmate, August 2008, 7-8

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Determination of Sulfide in Mining Leachates Using Ion Chromatography

Sulfide is an effective reagent for the precipitation of copper and other base metals in metal winning control. For an effective and economic operation of the SART Process (sulfidisation, acidification, recycling, thickening), monitoring the sulfide concentration is essential. Normally sulfide is determined by acidifying the sample and collecting the H_2S gas through a membrane in a buffer solution. The collected gas is then determined spectrophotometrically at 230 nm or after reaction with methylene blue at 600 nm. However, this offline method is very time-consuming and prone to interferences by other substances present.

According to **Metrohm** coupling a gas diffusion cell to an IC with subsequent spectrophotometric detection is an online alternative yielding faster and more accurate results. The H_2S gas derived from acidification of the sample enters a gas diffusion cell where it selectively diffuses through the hydrophobic membrane into a non-UV-absorbing acceptor solution. There it is deprotonated to the IC-compatible hydrogen sulfide anion (HS^-). Potentially interfering species cannot pass the membrane. Due to the selectivity of the gas diffusion cell and the direct ultraviolet absorption of the hydrogen sulfide anion at 230 to 250 mm (no post-column reagent is necessary), the overall analysis time is less than eight minutes.